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LMO-4 is a transcription factor, which exhibits prominent expression in epithelial cells, including that of the breast. Since previous members of the LIM only (LMO) gene family are oncogenes in lymphocytes, we hypothesized that LMO-4 may play a role in mammary gland development and cancer. We have now shown that expression of LMO-4 is associated with undifferentiated cellular stage of breast epithelial cells, such as that found during lobuloalveolar development in pregnancy and in breast cancer. Furthermore, the ErbB2 activator heregulin stimulates LMO4 expression in MCF-7 breast cancer cells, suggesting that LMO4 plays a role in this important oncogenic pathway. In protein interactions studies, we have found that LMO4 interacts with DNA-binding proteins, including GATA3. We have created transgenic mice in which we overexpress a dominant negative LMO-4 under the MMTV promoter (MMTV-engrailed-LMO4) to test the role of LMO-4 in mammary gland development. Our results show significant inhibition of lobuloalveolar development in these MMTV-engrailed-LMO4 transgenic mice, indicating that LMO-4 plays roles in proliferation and/or invasion of breast epithelial cells. Because these cellular features are associated with breast carcinogenesis and because LMO-4 is overexpressed in a subset of breast cancers, our studies implicate LMO4 as a possible oncogene in breast cancer.

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INTRODUCTION

Understanding the mechanisms involved in regulating proliferation and differentiation of breast epithelial cells is important for further understanding the causes, diagnosis and treatment of breast cancer. We identified a new protein called LMO-4, a member of a family of proteins that participate in gene regulation. Proteins belonging to this group have been shown to cause leukemia. We were therefore intrigued to find that LMO-4 is highly abundant in breast epithelial cells when these cells are proliferating. Our hypothesis is that LMO-4 is involved in regulation of proliferation and/or invasion of epithelial cells in normal breast and in breast cancer. To test this hypothesis, we are pursing the following **Specific Aims**:

#1. Define the expression pattern of LMO-4 during normal mouse breast development and in human breast cancer.

#2. Define the role of LMO-4 in normal breast development and in breast cancer, using a mouse transgenic approach.

#3. Identify and characterize protein partners for LMO-4 in human breast tissue.

BODY

***Objective #1.* Define the expression pattern of LMO-4 during normal mouse breast development and in human breast cancer.**

a. Raise and purify LMO-4 antisera.

This task has been accomplished as described in previous Progress Reports. In summary, we generated LMO4 antisera that are useful for Western blots but were not suitable for immunohistochemistry. After extensive characterization and effort to use these antibodies for developmental studies, we switched to using *in situ* hybridization with LMO4 cRNA probes and RNAse protection assays, as described in alternative approaches in our original proposal.

b. Obtain mouse embryos and pregnant mice at different developmental stages.

We have completed collecting fixed embedded mammary glands from mice at different developmental stages for use in immunohistochemistry and *in situ* hybridization studies. We have also completed isolation of RNA from mammary glands at different developmental stages for studying LMO-4 mRNA levels with RNAse protection assays.

c. Study LMO-4 expression during normal mammary gland development.

This task has been completed and results from RNAse protection assays and *in situ* hybridization studies were described in the last Progress Report, and are featured in our paper recently accepted for publication in *Oncogene* (see Appendix). The results show that LMO4 and Clim2 transcript levels are coordinately and greatly upregulated during mid-pregnancy, a stage in mammary gland development when epithelial cells are undergoing proliferation and invasion into the fat pad. Another expression peak is observed during lactation suggesting possible additional roles during this stage.

Our *in situ* hybridization studies also indicate that LMO-4 levels in mammary glands are highest during midpregnancy and become undetectable after weaning, and that LMO-4 is mainly expressed within the lobuloalveolar epithelial cells of the mammary gland. Together, these experiments correlate the expression of LMO-4 and Clim2 with a stage in development when

breast epithelial cells are relatively undifferentiated and undergoing proliferation and invasion. This expression pattern suggests the possibility that the LMO-4/Clim2 complex plays roles in maintaining proliferation, promoting invasion and/or suppressing differentiation – cellular features that characterize breast cancer cells.

d. Obtain human breast and breast tumor samples.

We have obtained a panel of cDNAs from breast tumor sections, which are spotted on a nylon membrane suitable for hybridization. These have now been used to study expression of LMO4 in human breast cancer cases.

e. Analyze expression of LMO-4 in normal human breast and breast cancer.

In the last Progress Report, we reported that that expression of LMO-4 and Clim2 varies markedly between three human breast cancer cell lines. LMO-4 is greatly overexpressed in one of the breast cancer cell lines, the MDA-MB-231 line, but low in MCF-7 cells. Transcript levels are not regulated by 17-β estradiol in MCF-7 cells. We now have tested the effect of heregulin, which is an ErbB2/Her2/Neu activator, on LMO4 expression in MCF-7 cells (Fig. 1). Interestingly, heregulin stimulates expression of LMO4 and this effect is partially blocked with an antibody to ErbB2/Her2/Neu, indicating that the effect is mediated via this receptor. These findings are exciting because they link LMO4 to an important oncogene in breast cancer; the ErbB2/Her2/Neu oncogene is overexpressed in 25% of breast cancer cases and predicts a poor prognosis.

Studies from other laboratories [1] have indicated that LMO4 is overexpressed in 50% of cases of breast cancer. Our own studies, using cDNA synthesized from human breast cancers indicates that the situation is more complex (Fig. 2). First, LMO4 levels vary widely in normal breast samples, and second, while LMO4 levels are high in breast cancers, the frequency of overexpression compared to normal breast is lower than 50% and probably more in the neighborhood of 20%.

f. Create and analyze stable MCF-7 breast cancer cell lines in which expression of LMO-4 and Clim2 can be induced. (New task)

This task was added to the original proposal based on a request dated November 13, 2002, which was granted by the Army Breast Program. The rationale for this task is to test directly whether LMO-4 overexpression contributes to the cancer phenotype of breast cancer cells. The approach for testing the role of LMO-4 in breast cancer is to introduce LMO-4 into breast cancer cells and ask what happens to gene expression and the biological behavior of breast cancer cells overexpressing LMO-4 and its partner Clim2.

We have completed the first part of this task, which was to create inducible MCF-7 cells expressing either LMO4 or Clim2. Initially, as reported in last year's Progress Report, we created these cells, using the Tet-on system. However, during long-term culturing, these cell lines did no have stable expression. We then switched to the Tet-off system and successfully created several MCF-7 cell line clones that express LMO4 (Fig. 3) and Clim2 (Fig. 4)in an inducible manner.

We have also made significant progress towards the second part of this task, which was to analyze global changes in gene expression in response to LMO4 and Clim2 expression. Microarray expression analyses have been successful in revealing functionally important signaling pathways in breast cancer [2] and may allow detection of alterations in cluster of genes

carrying out related functions [3, 4]. This approach was used to discover important functional pathways regulated by other factors, including BRCA1 [5, 6]. We have profiled expression in MCF-7 cells expressing LMO4 in a conditional manner, using the cell lines described in described above (Figs. 3 and 4). For these experiments, we selected 3 independent cell clones and profiled expression under basal conditions (control conditions, in the presence of doxycycline) and under induced conditions (LMO4 expression, 7 days after doxycycline withdrawal). To decrease variability, RNA samples from two independent experiments were pooled for each of the three cell clones. We hybridized to U133A Affymetrix chips, which contain 15,000 known or annotated human genes, and analyzed the data with the Cyber-T program, which was developed at UCI [7]. This statistical data package, which is especially suitable for pairwise comparisons, uses a Bayesian statistical framework to determine the local confidence (p-values) based on the *t*-test distribution of individual gene measurements. Thus, for each experimental condition, we can obtain: a mean expression level, a fold-change between control and experimental condition, and a *t*-value to establish the confidence level of the observed difference in expression of a particular gene between control and experimental conditions.

Using probability criteria of $p \leq 0.01$, 318 of 15,000 genes were altered after LMO4 induction. Of these 318 genes, 182 are upregulated and 136 are downregulated. Interestingly, this experiment suggests that LMO4 can not only stimulate gene expression, but it also can repress a group of genes. Table 1 shows a list of the top genes (listed in order of increasing p value) showing differential expression after induction of LMO4 in MCF-7 cells. Several of the genes regulated by LMO4 have been implicated in oncogenesis of epithelial cells, including breast cancer cells. Among these genes are: (1) The midkine/cytokine **Pleiotrophin (PTN),** which was purified from conditioned media of the highly malignant (also a high LMO4 expresser; Fig. 8A) MDA-MB-231 breast cancer cell line [8]. PTN is expressed in breast cancer and has been shown to act as an important paracrine and angiogenic factor for human breast cancer [9, 10]. It has growth-promoting and transforming activity on fibroblasts and epithelial cells, and mitogenic activity on endothelial cells [11]. Furthermore, PTN induces tube formation on endothelial cells and angiogenesis *in vitro* and in the rabbit corneal assay [10]. (2) **Metallothioneins 1X, -1L and –1H**, which are overexpressed in portion of breast cancer cases [12] and predict a poor prognosis [13]. Metallothioneins are thought to protect against oxidative stress and apoptosis [14]. (3) **FGF receptor 4,** which is overexpressed in breast cancer cell lines [15]. Several FGFs are known to be oncogenes in mouse mammary cancer [16]. At least one FGF, capable of activating FGFR-4, is overexpressed in human breast cancer [17]. (4) **Interleukin 8.** Its expression correlated with increased bone metastasis in a population of human breast cancer cells [18], and was demonstrated to stimulate osteoclastogenesis and bone resorbtion [19]. Moreover, IL-8 increases the invasiveness of ER-positive breast cancer cells by two fold, thus confirming the invasion-promoting role of IL-8 [20]. Further evidence for the important role of IL-8 in breast cancer comes from a study showing that combined administration of antibodies to human IL-8 and epidermal growth factor receptor results in increased antimetastatic effects on human breast carcinoma xenografts [21]. Additional up-regulated genes of interest are **BMP-7**, the **Ret proto-oncogene** and **Wnt-2B**. Among the selected down-regulated genes is **Tenascin X.** While its role in human breast cancer is unclear, tenascin X knockout mice exhibit increased tumor invasion and metastasis [22], consistent with a model in which tenascin inhibits migration and invasion. Additional down-regulated genes of interest are **Pleimorphic adenoma gene 1** and **Interleukin 17**.

In summary, LMO4 alters the expression of several genes involved in oncogenesis. We will now validate these targets using independent methods such as quantitative PCR and test whether Clim2 regulates the same panel of genes.

Ojective #2. **Define the role of LMO-4 in normal breast development and in breast cancer, using a transgenic approach.**

Two previously characterized members of the LMO-family, LMO-1 and LMO-2, have been found to be oncogenic. In humans these genes are overexpressed in lymphocytes due to fusion with the T-cell receptor in chromosomal translocations associated with acute lymphoblastic leukemia. These observations suggest that the LMO class of proteins plays roles in regulation of both proliferation and differentiation critical for organ development and that abnormalities in LMO-activity may lead to oncogenesis. Our hypothesis is that LMO-4 plays a role in normal breast development and that subversion of LMO-4 function or activity may contribute to formation of breast tumors.

We have elected to test our hypothesis using a transgenic approach, which allows us to test the role of LMO-4 in the context of the whole animal. We have made significant progress towards this goal. We have decided to create four sets of transgenic mice: one in which LMO-4 is overexpressed, one in which LMO-4 is converted into a "superactivator", one in which LMO-4 activity is inhibited, and a fourth one in which we have overexpressed a dominant negative form of the LMO-4-associated protein, CLIM.

a/b. Creation and testing of transgenic plasmids and microinjection of oocytes for establishing transgenic lines.

Mice for (1) overexpression of LMO-4, (2) overexpression of LMO-4/VP-16 activation domain fusion, (3) overexpression of LMO-4/engrailed repression domain fusion, and (4) overexpression of a dominant negative CLIM molecule: Transgenic lines have been established for all four lines. Analyses of mammary gland expression showed that we established 3 lines expressing MMTV-Engrailed-LMO4, 3 lines expressing MMTV-VP16-LMO4, and 2 lines expressing MMTV-dominant negative-Clim. For the MMTV-LMO4 line, we did not obtain any lines showing significant transgene expression. With the construction of the MCF-7 cells, which overexpress LMO4 in a conditional manner, we will be able to address the same question by alternative means, using these newly constructed cell lines (Fig. 3).

c. Breeding and analyses of transgenic lines.

The MMTV-LMO4 lines did not exhibit significant transgene expression in the mammary gland, thus preventing further analyses of this mouse strain. In contrast, we have been able to analyze mice from the other three transgenic lines. We have not observed a clear phenotype in the MMTV-VP16-LMO4 and MMTV-dominant negative-Clim lines. In contrast, we have obtained interesting data with the MMTV-Engrailed-LMO4 mice. To test the effect of the Engrailed-LMO4 molecule on mammary gland development, we placed it under control of the MMTV promoter (Fig. 5A), which directs high expression in epithelial cells of mammary glands in transgenic mice and has been extensively used for this purpose [23-26]. The fusion protein was HA tagged to allow its immunodetection in mammary glands. Of five transgenic lines, three independent lines expressed the transgene in mammary gland epithelial cells.

Expression of the transgene was found both in virgin and pregnant mammary glands (Fig. 5B) and was predominantly nuclear (Fig. 5C).

To evaluate the effects of expressing the Engrailed-LMO4 fusion protein in mammary gland epithelial cells, we examined mammary gland development by whole mount analyses in transgenic mice and compared them to wild-type littermates. Development of transgenic mammary glands of virgin mice was normal at 3 to 4 weeks (data not shown), but at 6 weeks a mild delay in the progression of ductal development was evident (Fig. 6). At 8 weeks, most transgenic mammary glands were normal (Fig. 6, compare WT panel and lower TG panel at 8 weeks), although we did observe occasional abnormality at that stage (Fig. 6, compare WT panel and upper TG panel at 8 weeks). These data indicate that the Engrailed-LMO4 fusion protein causes a transient delay in mammary gland development of virgin mice. In pregnant transgenic mice, a clear delay in alveolar development was evident at day 5.5 (Fig. 7A and B, left panels); this delay, however, was later overcome and by day 15.5, lobuloalveolar development was essentially normal (Fig. 7B, middle panels). No abnormalities were observed during lactation (Fig. 7B, right panels) and transgenic females were able to nurse normal-size litters. In conclusion, expression of the dominant negative Engrailed-LMO4 fusion protein in the mammary glands of mice results in the slowing of ductal development in virgin mice and a transient inhibition of alveolar development during pregnancy. These results are consistent with our hypothesis derived from the expression analyses and indicate that LMO4 is likely to play roles to promote invasion and/or proliferation of mammary gland epithelial cells.

Objective #3. **Identify and characterize protein partners for LMO-4 in human breast tissue.**

While LMO and LIM homeobox proteins are similar in that they are both localized to the nucleus, there is no evidence to suggest that the biological activity of LMOs is through direct DNA-binding. Insight into the biochemical mechanisms of actions for LMO proteins came from studies of LMO-1 and -2 in the hematopoietic system where it was found that LMO-2 interacts strongly with the bHLH domain of TAL1 [27] and GATA factors [28]. These proteins, as well as Clim-2, exist in a complex in erythroid cells. These experiments suggest a model in which LMO factors can be tethered to DNA by associating with DNA binding proteins, thus allowing the co-regulator CLIM to interact with transactivators that do not contain a covalently linked LIM domain.

We therefore propose that a LMO-4 and Clim-2 containing complex regulates gene activity in breast epithelial cells by associating with unidentified DNA-binding protein(s). The goal of the proposed experiments is to identify such factor(s). Specifically, we are interested in determining whether LMO-4 may interact with transcription factors or nuclear oncoproteins that have been shown to regulate differentiation and proliferation in normal and neoplastic breast.

a/b. Construction of yeast two hybrid libraries and screening with LMO-4 bait.

This task is already accomplished as described in the last Progress Report.

c. Characterization of potential positive interacting factors.

We have completed characterization of interacting factors isolated in the yeast two-hybrid screen as described in previous Progress Reports. For further characterization of interacting proteins, a new task, (d), has been added.

d. **To use immunoprecipitations of tagged LMO-4 and Clim2 proteins to identify potential interacting proteins.** (New task)

This task was added to the original proposal based on a request dated November 13, 2002, which was granted by the Army Breast Program. The LMO2 oncogene, which is highly related to LMO4 is known to interact with GATA factors [28]. This suggested the possibility that LMO4 might also interact with GATA factors. Of GATA factors, GATA3 has been shown to be expressed in breast cancer cells [29]. We therefore evaluated its expression during mammary gland development in the mouse (Fig. 8) and demonstrated that GATA3 is expressed throughout mammary gland development, and especially highly during pregnancy. In addition, we tested whether LMO4 is capable of interacting with GATA3, using co-immunoprecipitations of extracts from the MCF-7 cells stably expressing Myc tagged LMO4. In this assay, we were able to demonstrate an *in vivo* interaction between GATA3 and LMO4 (Fig. 9). In summary, LMO4 may act by associating with GATA3 in normal and malignant mammary epithelial cells. In future experiments, we plan to characterize this interaction further.

KEY RESEARCH ACCOMPLISHMENTS DURING LAST YEAR

1. Demonstration that LMO4 expression in breast cancer cells is regulated by the breast cancer oncogene ErbB2/Her2/Neu.
2. Demonstration that LMO4 is overexpressed in a subset of human breast cancers.
3. Obtaining stable MCF-7 cell lines that can be induced to express tagged LMO4 and Clim2.
4. Identifying several target genes of LMO4 using microarray analyses, many of which are involved in oncogenesis.
5. Showing that MMTV-Engrailed-LMO4 mice exhibit defective ductular development in virgin mice and defective alveolar development in pregnant mice.
6. Demonstrating expression of GATA3 in mammary glands of mice.
7. Demonstrating an in vivo interaction between LMO4 and GATA3.

REPORTABLE OUTCOMES TO DATE

1. Development of antisera
2. Transgenic mouse models for LMO expression
3. Permanent breast cancer cell lines expressing tagged LMO4 and Clim2 in a conditional manner
4. A fellowship award (BC000553) was funded based on work on this project. This grant from the Army Med Research & Development Command, entitled "Functional Analysis of LIM Domain Proteins and Co-Factors in Breast Cancer", supports Dr. Ning Wang. Total amount is $150,000 over three years.
5. Manuscript in press: Wang, N., Kudryavtseva, E., Chen, I., Sugihara, T.M., McCormick, J., and **Andersen, B.** 2003. Expression of an Engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice. *Oncogene,* in press.
6. Abstract: Wang, N., Kudryavstseva, E., Chen, I., Sugihara, T., & **Andersen, B.** 2002. The potential role of a new LIM factor, LMO4, in breast cancer. Proceedings Era of Hope meeting, Orlando Florida, September (Abstract P4-1).

7. Abstract: Wang, N., & **Andersen, B.** 2003. The potential role of a new LIM factor, LMO4, in breast cancer. California Breast Cancer Research Program meeting in San Diego, California, September.

CONCLUSIONS

In summary, we have made significant progress on all three specific aims. Our results show that LMO-4 expression is associated with undifferentiated breast epithelial cells such as those found during mid-pregnancy and in breast cancer. The major achievements during the last year include the finding that interfering with LMO4 in breast epithelial cells inhibits both ductular development in virgin mice and alveolar development in pregnant mice. An additional major achievement is the identification of several LMO4 target genes that are involved in oncogenesis and may explain the actions of LMO4. Collectively, our findings, some of which are reported in a paper in press (Oncogene) strengthen our hypothesis that overexpression of LMO4 may contribute to breast carcinogenesis. With our work, we hope to generate new ideas about treatment of breast cancer, thus impacting on reducing the human/economic cost of breast cancer

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APPENDIX COVER SHEET

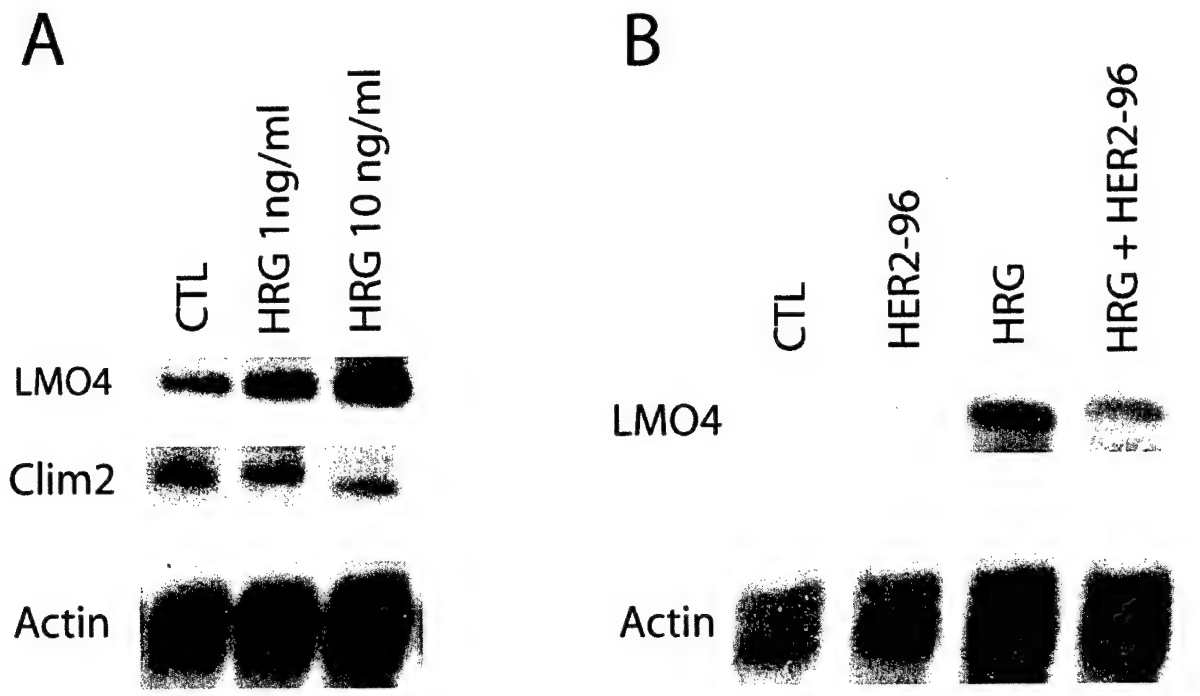

Figure 1. (a) *RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel) and β-actin (lower panel) in MCF-7 cells after heregulin treatment with the indicated concentrations for 24 hours.* ***(b)*** *RNAse protection assays showing expression of LMO4 (top panel) and β-actin (lower panel) in MCF-7 cells after treatment for 20 hours with heregulin and ErbB2 blocking antibody.*

Breast cancer profiling

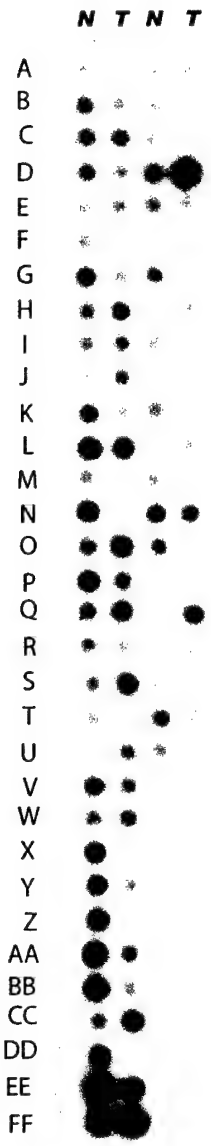

Figure 2. *Analyses of LMO4 expression in human breast cancer cases. cDNA synthesized from breast cancer RNA was spotted on a nylon membrane. The membrane was hybridized with a 32P-labelled probe specific for LMO4. For each tumor (T) sample there is a matched normal (N) sample from a normal region of the same breast. For example, in row D there are samples from two patients and the patient represented in the two rows to the right exhibits striking overexpression of LMO4.*

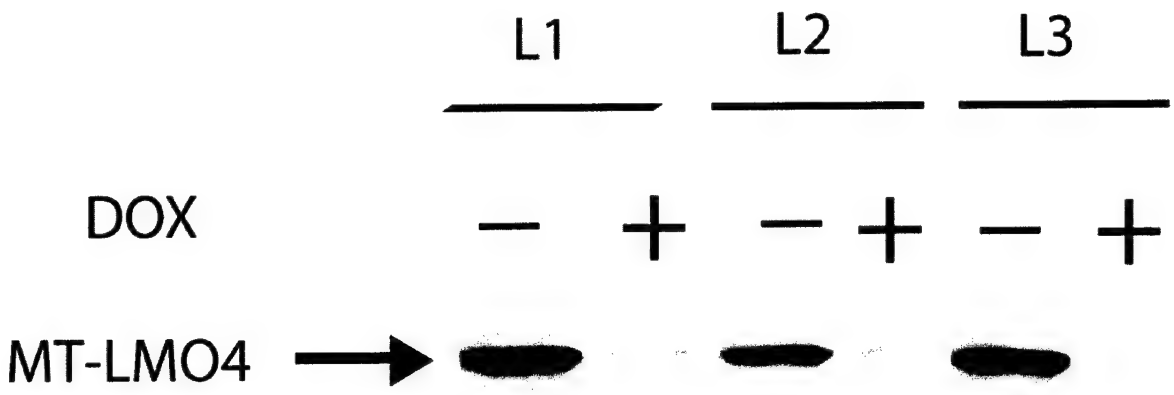

Figure 3. *Representative Tet-Off LMO4-inducible MCF-7 cell clones. Shown are three independent MCF-7 cell clones, with and without doxycycline (DOX) analyzed by a western blot, using Myc antibody.*

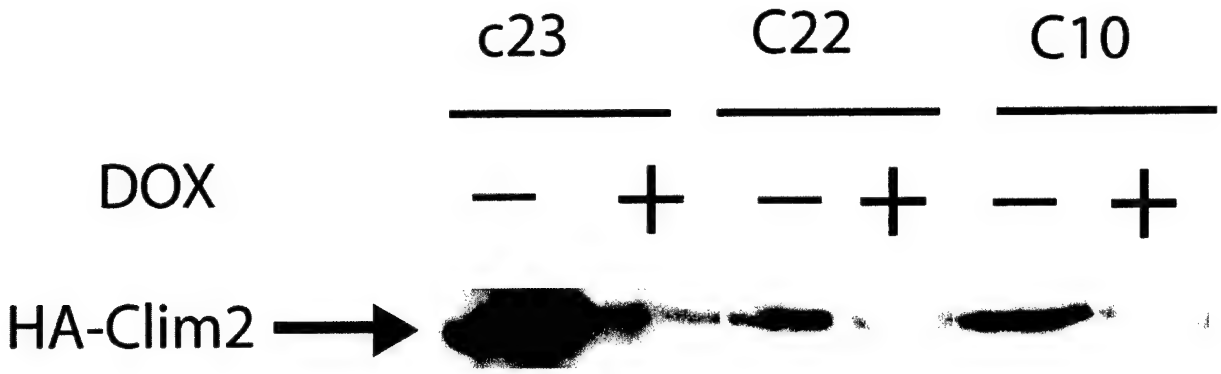

Figure 4. *Representative Tet-Off MCF-7 cell clones. Shown are three independent Clim2-inducible MCF-7 cell clones, with and without doxycycline (DOX) analyzed by a western blot, using HA antibody.*

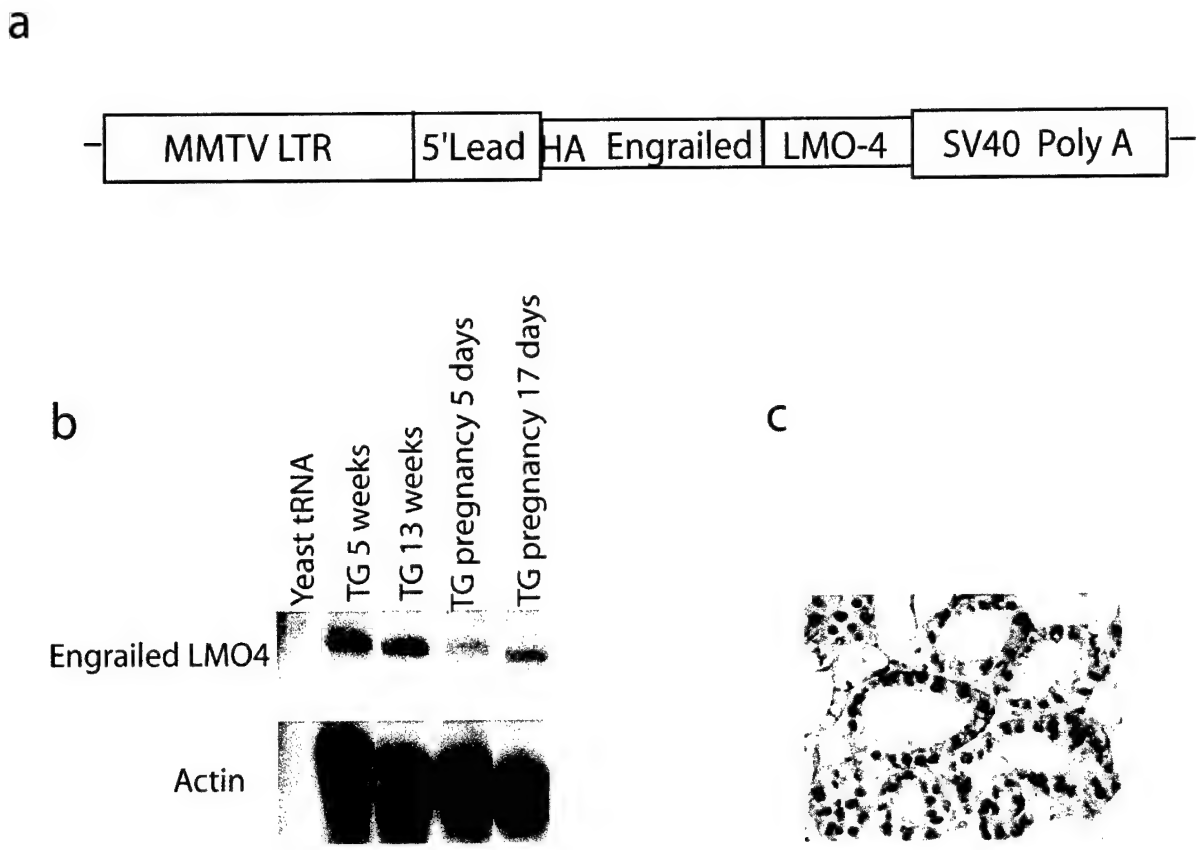

Figure 5. *The Engrailed-LMO4 transgene.* ***(a)*** *A schematic of the transgene.* ***(b)*** *RNAse protection assays showing expression of the Engrailed-LMO4 transgene at the indicated developmental time points.* ***(c)*** *HA immunostaining of mammary gland (day 1 of lactation) from MMTV-HA-Engrailed-LMO4 mice.*

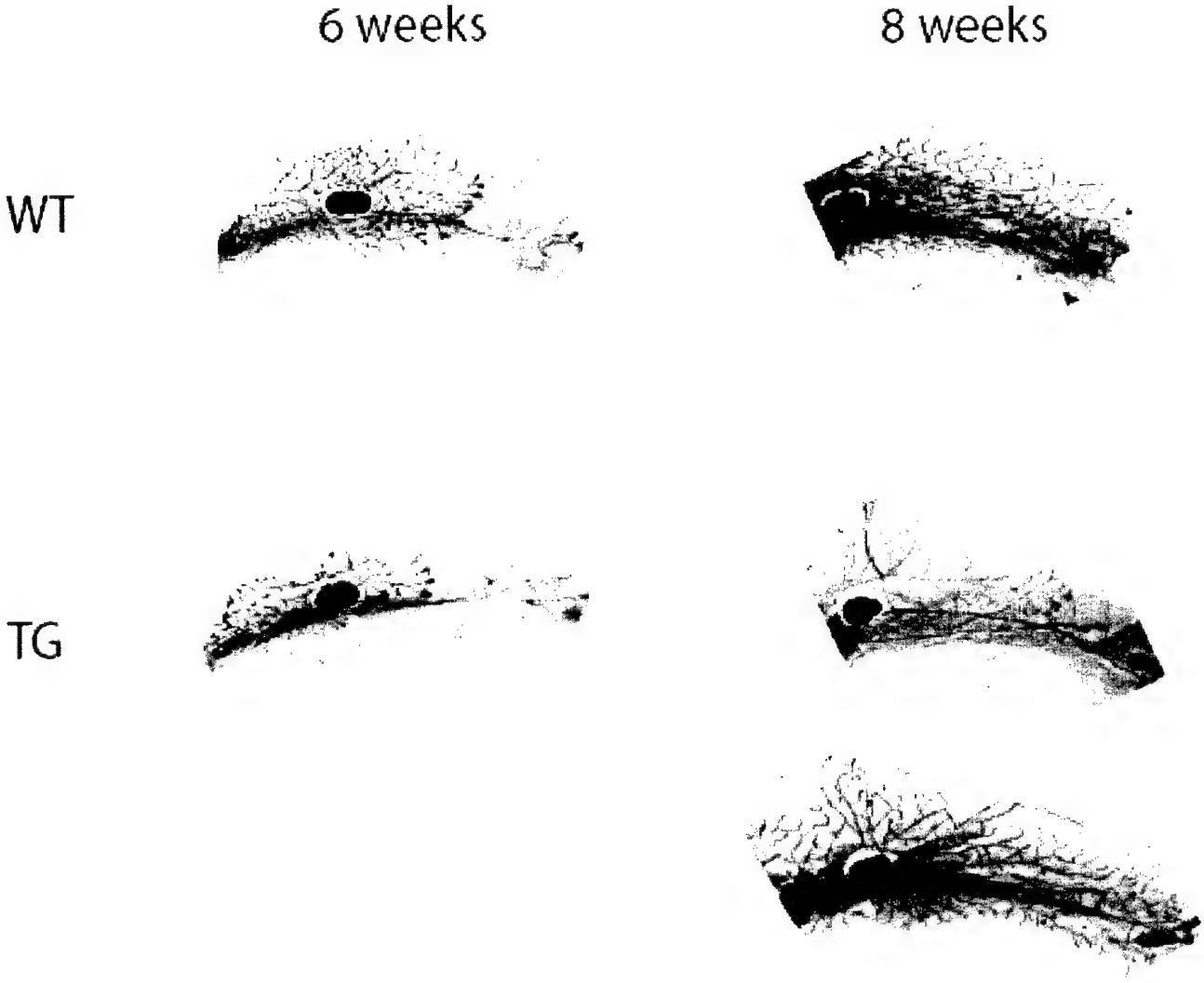

Figure 6. *The effect of the Engrailed-LMO4 fusion protein on mammary gland development in virgin mice. Whole mount staining of the fourth inguinal mammary glands from MMTV-HA-Engrailed-LMO4 (TG) mice and littermate wild-type (WT) controls at the indicated developmental stages.*

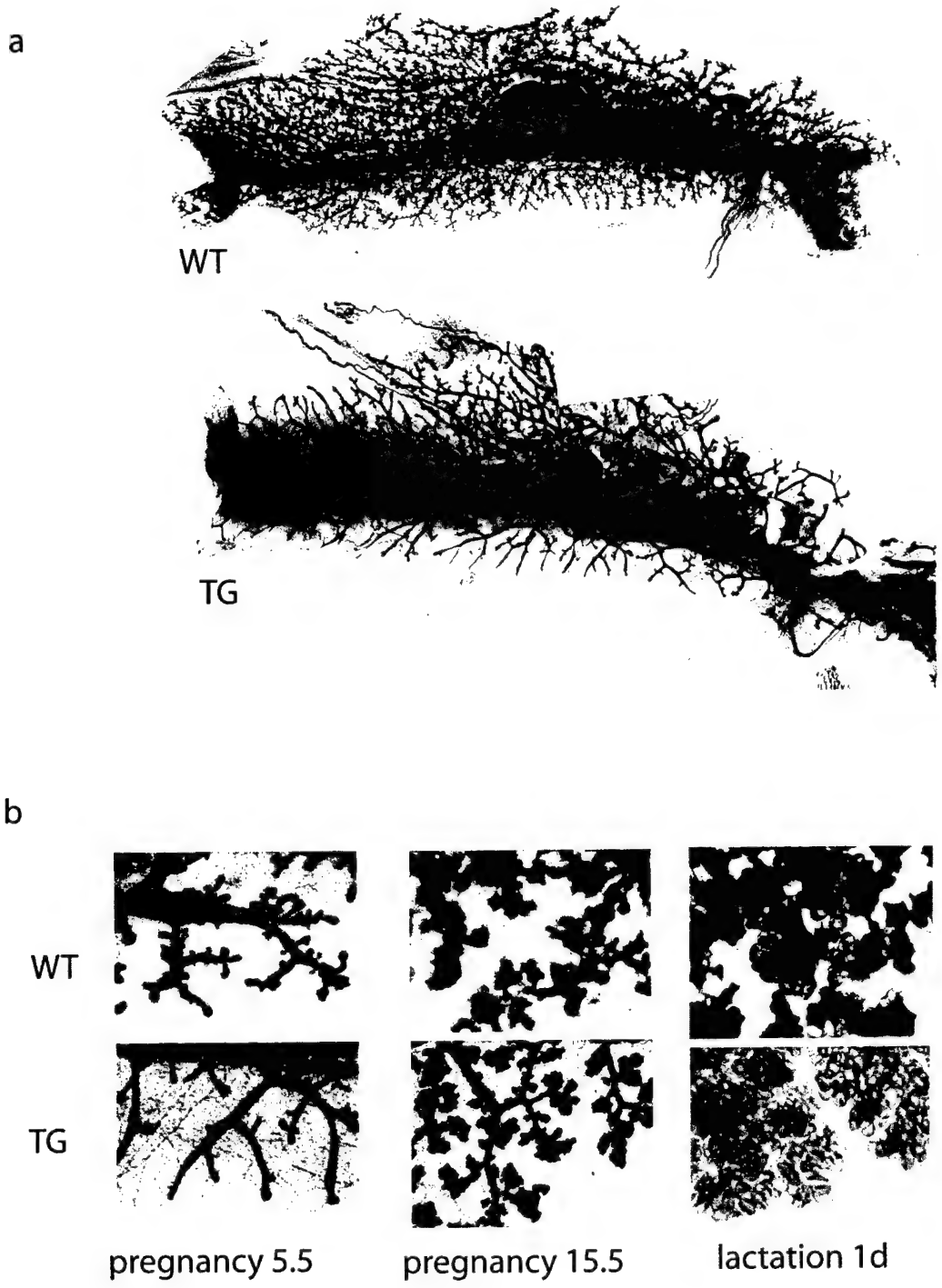

***Figure** 7. The effect of the Engrailed-LMO4 fusion protein on mammary gland development during pregnancy. **(a)** Whole mount overview of mammary glands from 5.5 day pregnant mice comparing wild-type (WT) and transgenic (TG) mice. **(b)** Whole mount analyses in higher magnification from the indicated developmental stages.*

Figure 8. *Expression of GATA3 in mammary glands from the indicated stages, using RNAse protection assays.*

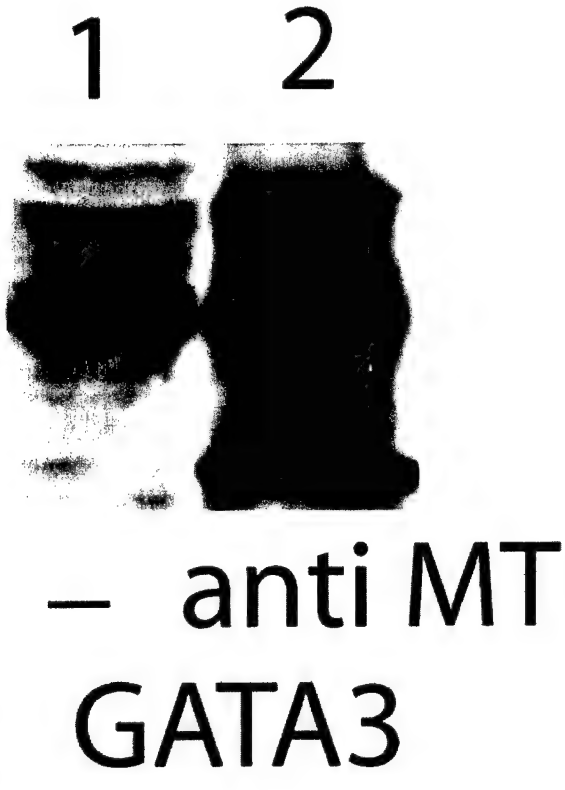

Figure 9. Co-immunoprecipitation showing interaction between LMO4 and GATA3 in MCF-7 breast cancer cells. Extracts from MCF-7 cells expressing Myc tagged LMO4 were analyzed by SDS gel electrophoreses without (lane 1) or after immunoprecipitation with a Myc antibody (lane 2). Western blot was probed with GATA3 antibody. Immunoprecipitation with Myc antibody precipitates GATA3, indicating interaction between LMO4 and GATA3.

Expression of an Engrailed-LMO4 fusion protein in mammary epithelial cells inhibits mammary gland development in mice

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Summary

LIM domain factors and associated co-factors are important developmental regulators in pattern formation and organogenesis. In addition, overexpression of two LIM-only factors (LMOs) causes acute lymphocytic leukemia. The more recently discovered LMO factor LMO4 is highly expressed in proliferating epithelial cells, and frequently overexpressed in breast carcinoma. Here we show that while LMO4 is expressed throughout mammary gland development, it is dramatically up-regulated in mammary epithelial cells during mid-pregnancy. The LMO co-activator Clim2/Ldb1/Nli showed a similar expression pattern, consistent with the idea that LMO4 and Clim2 act as a complex in mammary epithelial cells. In MCF-7 cells, LMO4 transcripts were up-regulated by heregulin, an activator of ErbB receptors that are known to be important in mammary gland development and breast cancer. To test the hypothesis that LMO4 plays roles in mammary gland development, we created an Engrailed-LMO4 fusion protein. This fusion protein maintains the ability to interact with Clim2, but acts as a dominant repressor of both basal and activated transcription when recruited to a DNA regulatory region. When the Engrailed-LMO4 fusion protein was expressed under control of the MMTV promoter in transgenic mice, both ductular development in virgin mice and alveolar development in pregnant mice were inhibited. These results suggest that LMO4 plays a role in promoting mammary gland development.

The LIM motif, a cysteine-rich zinc-coordinating domain that mediates protein-protein interactions, was originally discovered as a component of homeodomain transcription factors (reviewed in Bach, 2000). A second class of LIM domain transcription factors, composed almost entirely of two tandem LIM domains, is referred to as LIM-only (LMO) proteins. Two members, LMO1 and LMO2, are oncoproteins found at sites of chromosomal translocations in acute T-cell leukemia (Rabbitts et al., 1999). LMO proteins do not bind DNA directly, but regulate gene transcription by associating with other transcription factors. This model is supported by studies showing that LMOs, through their LIM domains, exist in a stable complex with helix loop helix (HLH) heterodimeric partner proteins that include TAL1(SCL)/E12 (Bao et al., 2000; Herblot et al., 2000; Larson et al., 1996; Mead et al., 2001; Ono et al., 1998; Osada et al., 1995; Osada et al., 1997; Valge-Archer et al., 1994; Visvader et al., 1997; Wadman et al., 1994; Wadman et al., 1997), and GATA factors (Mead et al., 2001; Ono et al., 1998; Osada et al., 1995; Wadman et al., 1997). In addition, LIM domains of LIM homeodomain and LMO proteins interact strongly with cofactors, including the coactivators Clim1 and Clim2/Lbd1/NLI (Agulnick et al., 1996; Bach et al., 1997; Bach et al., 1999; Jurata et al., 1996; Visvader et al., 1997), which confer transcriptional activation and promote synergism between DNA-binding proteins (Bach, 2000).

Based on the prominent expression of Clim2 in proliferating epithelial cells of the epidermis and hair follicles, we discovered LMO4 as a Clim2 interacting protein in the epidermis (Sugihara et al., 1998). LMO4, simultaneously discovered by other laboratories (Grutz et al., 1998; Kenny et al., 1998), is the main LIM domain factor expressed in proliferating epithelial cells of the epidermis and hair follicles (Sugihara et al., 1998). Interestingly, the human *LMO4* gene was

initially cloned from a breast cancer cDNA library (Racevskis et al., 1999), and subsequent studies showed it to be overexpressed in more than half of all invasive breast carcinomas (Visvader et al., 2001). Furthermore, LMO4 and Clim2 overexpression interfered with differentiation of cultured mammary epithelial cells (Visvader et al., 2001).

The goals of our studies were to establish a dominant negative LMO4 molecule that can be used to repress transcription of LMO4 target genes and to study the biological function of LMO4 in the mammary gland *in vivo*. We show that fusion of the repression domain from the *Drosophila* Engrailed homeobox protein (Han & Manley, 1993) to LMO4 creates a strong transcriptional repressor, capable of interfering with basal and activated transcription. Expression of this fusion molecule under the MMTV promoter in mammary glands of transgenic mice leads to inhibition of ductular and alveolar development, suggesting that LMO4 is involved in progression of mammary gland development.

LMO4 is up-regulated in mammary epithelial cells during mid-pregnancy and by heregulin in MCF-7 breast cancer cells: To gain insights into the role of LMO4 in mammary gland biology, we assessed its expression in mouse mammary gland and breast cancer cell lines. In contrast to a previous study employing Northern blot analyses on total RNA (Visvader et al., 2001), the sensitive RNAse protection assays show that LMO4 transcripts are easily detected in virgin mammary glands and that expression levels remain relatively stable from age 4 weeks to 14 weeks (Fig. 1a). However, there is dramatic up-regulation of LMO4 in mammary glands from mid-pregnancy, with levels falling late in pregnancy (Fig. 1a), and a moderate increase in LMO4 levels during early lactation (Fig. 1a). Clim2 levels are coordinately regulated during mammary

gland development, with highest levels found in mid-pregnancy (Fig. 1a), consistent with the idea that LMO4 and Clim2 act as a complex. *In situ* hybridization studies on mammary gland sections show that LMO4 is primarily expressed in ductular and alveolar epithelial cells (Fig. 1b). Consistent with the RNAse protection assay experiments, LMO4 levels are high at day 14.5 and lower at day 18.5 (Fig. 1b). The surge in LMO4/Clim2 transcript levels during mid-pregnancy suggests an especially important function at this developmental stage characterized by dramatic epithelial cell proliferation and stromal invasion.

In three different human breast cancer cell lines, LMO4 transcript levels vary from high in the estrogen receptor negative MDA-MB-231, intermediate in the estrogen receptor negative MDA-MB-453, to low in the estrogen receptor positive MCF-7 (Fig. 1c). Estradiol did not increase LMO4 expression in MCF-7 cells (Fig. 1c), consistent with findings in human breast cancer indicating that LMO4 is especially characteristic for estrogen receptor negative tumors (Gruvberger et al., 2001). In contrast to the coordinately regulated expression of LMO4 and Clim2 during normal mammary gland development (Fig. 1a), Clim2 levels remain constant in breast cancer cell lines that express high levels of LMO4 transcripts (Fig. 1c) and protein (Fig. 1d). These results suggest that relative overexpression of LMO4 compared to Clim2 may be important for LMO4 actions in breast cancer. Because LMO4 may be localized to the cytoplasm under certain conditions (Kenny et al., 1998), we evaluated its cellular distribution in breast cancer cells by generating MCF-7 cells stably expressing a myc tagged (MT) LMO4. In these cells, LMO4 is restricted to the nucleus (Fig. 1e). In contrast to the lack of estrogen regulation, LMO4 expression is stimulated by the ErbB ligand heregulin, which is known to be important for alveolar maturation and proliferation (Fig. 1f). Heregulin is thought to act through ErbB2-

containing heterodimers (Stern, 2003) and its effect was partially blocked by an ErbB2 antibody (Fig. 1g), suggesting a role for ErbB2 in heregulin-mediated up-regulation of LMO4.

The observation that LMO4 may be downstream of heregulin/ErbB2 is consistent with findings that the mesenchymally-expressed heregulin α, like LMO4, is strikingly upregulated in mid-pregnancy (Yang et al., 1995). In addition, heregulin and the ErbB2/ErbB3/ErbB4 receptors, which have growth-stimulatory roles (Aguilar et al., 1999; Krane & Leder, 1996), are particularly important for alveolar morphogenesis (Jones et al., 1996; Jones & Stern, 1999; Jones et al., 1999; Li et al., 2002; Yang et al., 1995). ErbB2 is also overexpressed in 15 – 40 % of breast cancer cases where it is associated with increased invasiveness and metastasis, as well as poor prognosis (Eccles, 2001; Slamon et al., 1989). Our findings suggest the possibility that LMO4 may participate in heregulin/ErbB signaling in the mammary gland.

An Engrailed-LMO4 fusion protein is capable of protein-protein interactions and acts as a strong transcriptional repressor: LMO4 forms a complex with Clim co-activators in epithelial cells and is thought to be recruited to DNA-binding proteins, resulting in transcriptional activation of target genes. We hypothesized that fusing the *Drosophila* Engrailed transcriptional repression domain to LMO4 would create a dominant negative molecule capable of suppressing LMO4 target genes (Fig. 2a). When fused to heterologous transcription factors, the Engrailed repression domain confers strong transcriptional repression. This quality was successfully used to obtain insights into the biological function of a spectrum of transcriptionally active molecules, including c-Myb (Taylor et al., 1996), *Xenopus* tailless (Hollemann et al., 1998), GATA factors

(Dasen et al., 1999; Liu et al., 2002; Sykes et al., 1998), homeobox factors iroquois3 (Kudoh & Dawid, 2001) and RaxL (Chen & Cepko, 2002), and β-catenin (Montross et al., 2000).

To test whether the Engrailed-LMO4 fusion protein was capable of protein-protein interactions, we performed co-immunoprecipitation assays in HEK293T cells transfected with expression plasmids encoding tagged LMO4 and Clim2, as well as fusion proteins with LMO4 and Clim2. As expected, Clim2 antiserum precipitated LMO4 in cells transfected with Clim2 and LMO4 (Fig. 2b). In cells co-transfected with tagged VP16-Clim2 and Engrailed-LMO4 fusion proteins, both proteins could be precipitated independent of whether the precipitating antibody was directed against VP16-MT-Clim (Fig. 2c, left panel) or HA-Engrailed-LMO4 (Fig. 2c, right panel). We conclude that the fusion of the *Drosophila* Engrailed repression domain to LMO4 does not interfere with its ability to interact with Clim proteins.

Because natural target genes for LMO4 are unknown, we tested the effectiveness of the Engrailed-LMO4 fusion in a GAL reporter system where we monitored the transcriptional activity of a luciferase reporter gene under the control of GAL DNA-binding sites and a minimal promoter (Sugihara et al., 1998) (Fig. 2d). While LMO4-GAL (Fig. 2d, panel 2) has little effect on basal activity of the promoter, Engrailed-LMO4-GAL (Fig. 2d, panel 3) represses transcription of the reporter gene 29-fold. Furthermore, Engrailed-LMO4-GAL could completely overcome a 105-fold activation conferred by the recruitment of a Clim-VP16 fusion protein (Fig. 2d, compare panels 5 and 6). Clim alone is a weak activator in this system and the Clim-VP16 fusion protein is used because the viral VP16 transactivation domain can confer strong transactivation to heterologous proteins. In summary, these experiments suggest that an

Engrailed-LMO4 fusion protein can repress both basal and activated expression of LMO4 target genes, and that this fusion molecule may be useful to test the biological functions of LMO4.

Expression of the Engrailed-LMO4 fusion protein in mammary gland epithelial cells of mice interferes with mammary gland development: To test the effect of the Engrailed-LMO4 molecule on mammary gland development, we placed it under control of the MMTV promoter (Fig. 3a), which has been extensively used to direct high expression in epithelial cells of mammary glands in transgenic mice (Guy et al., 1992; Kitsberg & Leder, 1996; Krane & Leder, 1996; Muller et al., 1988). Three independent lines expressed the transgene in mammary gland epithelial cells. Expression of the transgene was found both in virgin and pregnant mammary glands (Fig. 3b) and by immunohistochemistry with an HA antibody expression was predominantly nuclear (Fig. 3c). The relatively constant level of the transgene expression (Fig. 3b) is probably because the transgene in this line is upregulated at the very end of pregnancy as has been described for other MMTV transgenic mice (Jones et al., 1999). We examined mammary gland development by whole mount analyses in transgenic mice and compared them to wild-type littermates. Development of transgenic mammary glands of virgin mice was normal at 3 to 4 weeks (data not shown), but at 6 weeks a mild delay in the progression of ductal development was evident (Fig. 3d). At 8 weeks, most transgenic mammary glands were normal (Fig. 3d, lower TG panel at 8 weeks) although we did observe occasional abnormality at that stage (Fig. 3d, upper TG panel at 8 weeks). These data indicate that the Engrailed-LMO4 fusion protein causes a transient delay in mammary gland development of virgin mice.

In pregnant transgenic mice, a clear delay in alveolar development was evident at day 5.5 (Fig. 4a and b); this delay, however, was later overcome and by day 15.5, lobuloalveolar development was essentially normal (Fig. 4b). No abnormalities were observed during lactation (Fig. 4b) and transgenic females were able to nurse normal size litters. In conclusion, expression of the dominant negative Engrailed-LMO4 fusion protein in the mammary glands of mice results in the slowing of ductal development in virgin mice and a transient inhibition of alveolar development during pregnancy, suggesting that LMO4 plays roles in both ductular and alveolar development *in vivo*.

The phenotype of the MMTV-HA-Engrailed-LMO4 mice may be distinct from the expected phenotype of LMO4 null mice. First, the Engrailed-LMO4 fusion protein can suppress the expression of LMO4 target genes both under basal and activated conditions. In contrast, deletion of the LMO4 gene is likely to affect only the genes where LMO4 is actually participating in regulated transcription. Second, while it is generally thought that LMOs in combination with Clims are involved in transactivation, LMO4 may also participate in repression of certain genes as has been suggested with BRCA1-mediated transcriptional activity (Sum et al., 2002). The Engrailed-LMO4 fusion protein would not be expected to affect these genes since they are already repressed. Finally, LMO4 may also act by binding to and sequestering other proteins in solution, a process the Engrailed-LMO4 fusion protein would not be expected to inhibit. Such mechanisms have been proposed for the effect of *Drosophila* lmo in the fly wing (Zeng et al., 1998).

The effect of Engrailed-LMO4 expression in mammary glands was most clearly observed in early pregnancy but the defect was overcome towards the end of pregnancy. Such defects, in which mammary gland development is slowed but not blocked, have been previously described in other genetically modified mice such as those with mutations in the ErbB2 gene (Stern, 2003). However, it is not possible to conclude that the role of LMO4 is restricted to ductular development in virgin mice and alveolar development in early pregnancy because it is impossible to determine which levels of transgene expression are required to block endogenous LMO4 protein levels. Despite these limitations of the dominant negative approach, our results strongly support roles for LMO4 in both ductular and alveolar development. Moreover, the dominant negative LMO4 is a promising tool to evaluate the possible role of LMO4 in signaling pathways and in breast cancer.

The etiology of sporadic breast cancers is multifactorial and thought to involve stepwise mutations in several oncogenes and tumor suppressor genes. The findings described in this paper are of importance because there are parallels between mammary epithelial cells during pregnancy and in breast cancer, and the LMO4 gene is frequently overexpressed in breast cancer. While neoplastic breast epithelial cells clearly have properties distinct from epithelial cells of the developing breast, the two also share similarities such as active proliferation and lack of terminal differentiation (Rudland et al., 1998). Our studies -- showing high expression of LMO4 during a stage in mammary gland development when there is active proliferation and stromal invasion, and the inhibition of these processes with a dominant negative LMO4 molecule -- lend support for the idea that LMO4 up-regulation may contribute to the tumorigenic characteristics of mammary epithelial cells (Visvader et al., 2001).

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Figure legends

Figure 1. Expression of LMO4 and Clim2 during mammary gland development and in response to regulators of mammary gland development. **(a)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel) and β-actin (bottom panel) during the indicated stages of mammary gland development. **(b)** *In situ* hybridization study showing expression of LMO4 in mammary glands at day 14.5 of pregnancy (left panel), day 18.5 of pregnancy (middle panel) and day 10 after weaning (right panel). ^{35}S labeled cRNA probes specific for mouse LMO4 were applied to formalin fixed tissue as described (Sugihara et al., 1998). **(c)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel) and β-actin (lower panel) in the indicated breast cancer cell lines and with estradiol (E2) treatment (20 ng/ml) for the indicated times. MCF-7 cells were grown in the presence of phenol red-free media and charcoal stripped serum. **(d)** Western blot of whole cell extracts from MDA-MB-231 and MCF-7 cells, using rat LMO4 antibody (Sum et al., 2002) and rabbit Clim antisera (Bach et al., 1999). **(e)** Immunolocalization LMO4 in MCF-7 cells stably expressing myc tagged LMO4. After fixing with formalin, slides were incubated with a myc antibody and signal detected with peroxidase. **(f)** RNAse protection assays showing expression of LMO4 (top panel), Clim2 (middle panel) and β-actin (lower panel) in MCF-7 cells after heregulin treatment with the indicated concentrations for 24 hours. MCF-7 cells were maintained in serum-free media. Similar effects were observed after 48 hour treatment (data not shown). **(g)** RNAse protection assays showing expression of LMO4 (top panel) and β-actin (lower panel) in MCF-7 cells after treatment for 20 hours with heregulin and ErbB2 blocking antibody (Clone Her2-96, Sigma).

RNA isolation and RNAse protection assays were carried out as previously described (Andersen et al., 1997), using ^{32}P-labeled cRNAs specific for mouse and human LMO4, Clim2 and β-actin.

Figure 2. Interactions between Clim2 and LMO4 fusion proteins. **(a)** A model for the activity of the Engrailed-LMO4 fusion protein. Under normal condition (left panel), LMO4/Clim complexes are thought to be recruited to promoters of target genes by associating with DNA-binding proteins (indicated as X), resulting in transactivation. Recruitment of the Engrailed-LMO4 fusion proteins to the same complexes should result in transcriptional repression. **(b)** Immunoprecipitation of cell lysates from HEK293T cells transfected with expression plasmids encoding myc tagged (MT) LMO4 and Clim2, using IgG (lane 1) and Clim antibody (Bach et al., 1999) (lane 2). Western blot was probed with MT antibody (Invitrogen). **(c)** Immunoprecipitation of cell lysates from HEK293T cells (Sugihara et al., 2001) transfected with expression plasmids encoding HA tagged Engrailed-LMO4 and myc tagged VP16-Clim2, using IgG (lanes 1 and 3), MT antibody (lane 2) and HA antibody (lane 4). Western blots were probed with the indicated antibodies. The HA-Engrailed-LMO4 fragment was generated in the mammalian expression vector pCMX by fusing the repression domain representing amino acids 2 – 299 of the Drosophila Engrailed gene (Han & Manley, 1993) to an HA tag at the N-terminus and the full length LMO4 coding sequence at the C-terminus. The pCMXGAL-LMO4 and pCMXGAL-EngrailedLMO4 plasmids contain the full-length LMO4 cDNA and the HA-Engrailed-LMO4 fusion protein linked to the GAL DNA-binding domain. The pCMXVP16-Clim plasmid contains the C-terminal LIM-interaction domain of Clim1 (Bach et al., 1999) linked to the VP16 transactivation domain. **(d)** The indicated GAL DNA-binding domain fusion proteins and VP16 fusion proteins were transfected into HEK293T cells with a GAL-luciferase

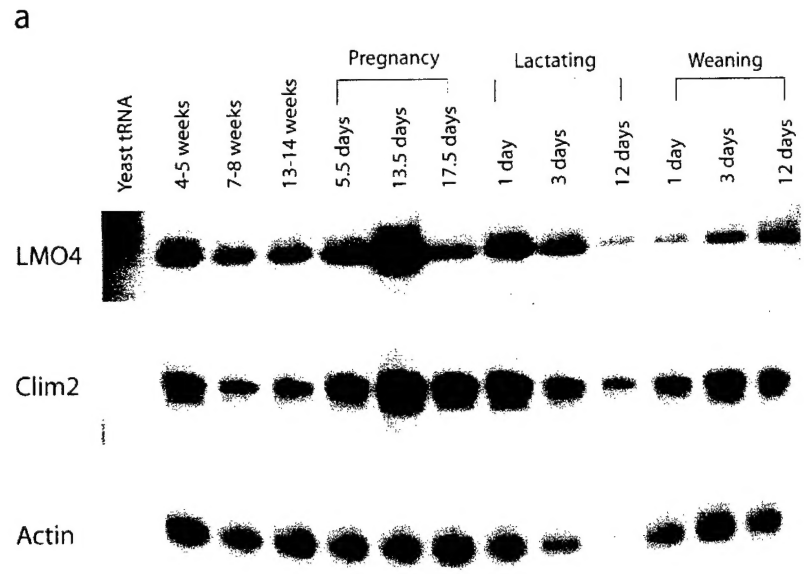
reporter plasmid, using calcium-mediated gene transfer (Sugihara et al., 1998). The results, expressed as light units, represent the mean and standard deviation from triplicate transfections. IP, immunoprecipitation; W, Western blot.

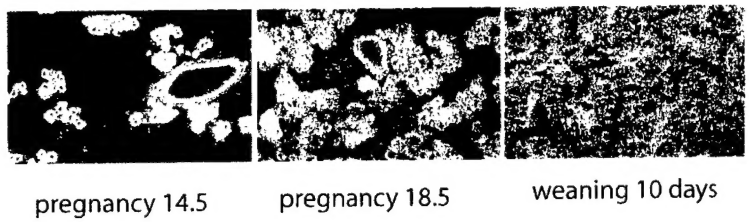
Figure 3. The effect of the Engrailed-LMO4 fusion protein on mammary gland development in virgin mice. **(a)** A schematic of the transgene. The MMTV-HA-Engrailed-LMO4 plasmid was created by cloning the HA-Engrailed-LMO4 fragment into the EcoR1 site of the MMTV-SV40-BSSK plasmid (Leder et al., 1986). To generate transgenic mice, the plasmid was cut with Xho1 and Spe1 to remove extraneous sequences, and the purified DNA fragment was then injected into fertilized CB6F1 oocytes, which were implanted into pseudopregnant mice. Of 13 mice born, five contained the MMTV-HA-Engrailed-LMO4 sequences as assessed by PCR with oligonucleotides specific for MMTV sequences. Of these five lines of founder mice, three (lines #1, 2 and 7) expressed the transgene as assessed by immunohistochemistry with HA antibody on pregnant mammary glands. The three expressing lines were expanded by breeding into CB6F1 mice. Experiments were carried out with transgenic mice derived from lines #1, 2 and 7, which showed a comparable level of abnormality in mammary gland development. **(b)** RNAse protection assays showing expression of the Engrailed-LMO4 transgene from line #7 at the indicated developmental time points. The probe, which corresponded to the *Drosophila* Engrailed part of the fusion molecule, was specific because no signal was observed in mammary glands from wild-type mice (data not shown). **(c)** HA immunostaining of mammary gland (day 1 of lactation) from MMTV-HA-Engrailed-LMO4 mice. Immunostaining of wild-type littermates gave no staining with the HA antibody (data not shown) indicating that the staining is specific. The mammary glands were fixed for one hour at room temperature in a solution composed of 6

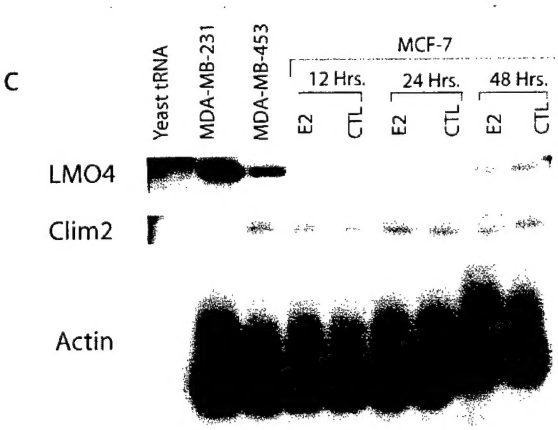
parts ethanol, 3 parts water and 1 part formaldehyde, followed by storage in 70% ethanol at 4° C. Paraffin embedded tissue sections were stained with a monoclonal HA antibody (Covance) using peroxidase. **(d)** Whole mount staining of the fourth inguinal mammary glands from MMTV-HA-Engrailed-LMO4 (TG) mice and littermate wild-type (WT) controls at the indicated developmental stages. Shown are representative results from analyses of 16 (6 weeks) and 3 (8 weeks) TG mice. The mammary glands were dissected, processed as a whole mount, fixed and stained with hematoxylin as described (Brisken et al., 1999), and photographed at the same magnification.

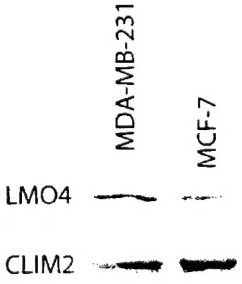
Figure 4. The effect of the Engrailed-LMO4 fusion protein on mammary gland development during pregnancy. **(a)** Whole mount overview of mammary glands from 5.5 day pregnant mice comparing wild-type (WT) and transgenic (TG) mice. **(b)** Whole mount analyses in higher magnification from the indicated developmental stages. All magnifications are the same. Shown are representative results from the analyses of 10 (5.5 day), 6 (15.5) and 4 (lactating) transgenic mice.

Fig. 1

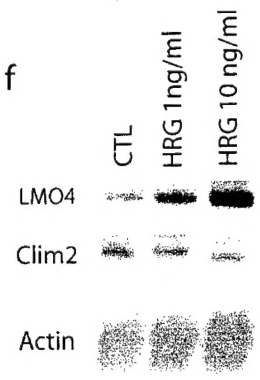

a
Pregnancy
Lactating
Weaning
Yeast tRNA
4-5 weeks
7-8 weeks
13-14 weeks
5.5 days
13.5 days
17.5 days
1 day
3 days
12 days
1 day
3 days
12 days
LMO4
Clim2
Actin


b
pregnancy 14.5
pregnancy 18.5
weaning 10 days


c
Yeast tRNA
MDA-MB-231
MDA-MB-453
MCF-7
12 Hrs.
24 Hrs.
48 Hrs.
E2
CTL
E2
CTL
E2
CTL
LMO4
Clim2
Actin


d
MDA-MB-231
MCF-7
LMO4
CLIM2


e


f
CTL
HRG 1ng/ml
HRG 10 ng/ml
LMO4
Clim2
Actin

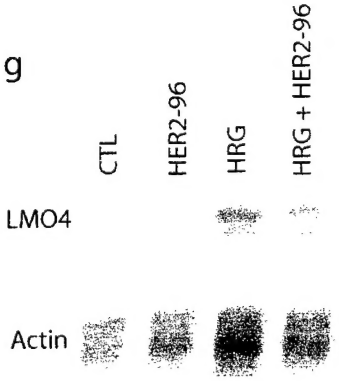
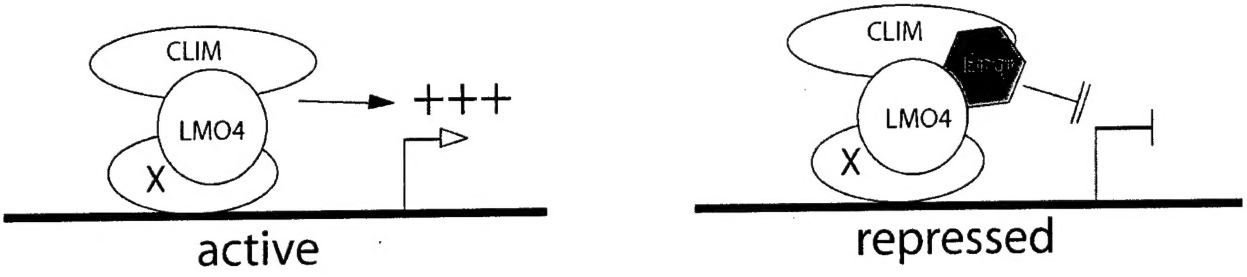

g
CTL
HER2-96
HRG
HRG + HER2-96
LMO4
Actin

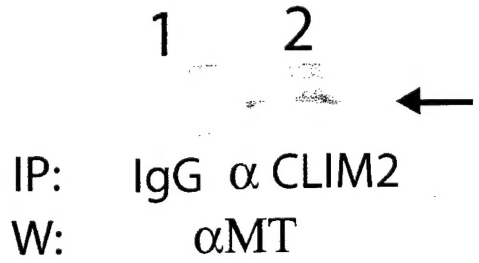
Fig. 2

a



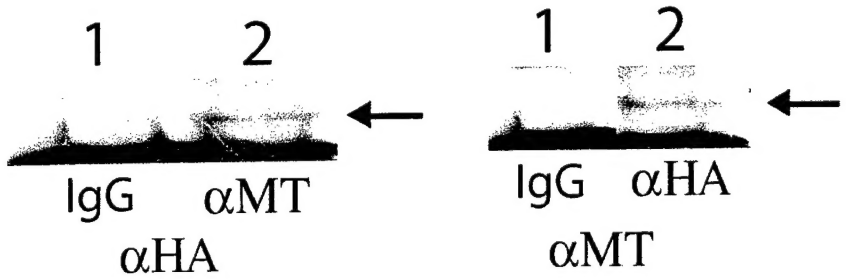
b

pCS2MT-LMO4
pcDNA3-CLIM2



c

pCMX-VP16-MT-CLIM2
pCMX-HA-Engrailed-LMO4



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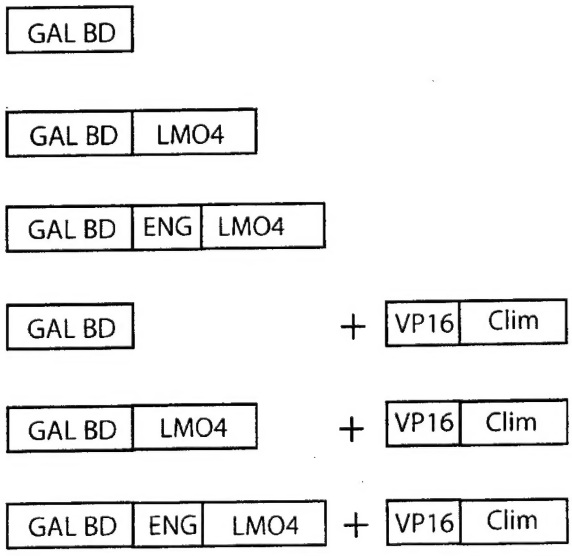


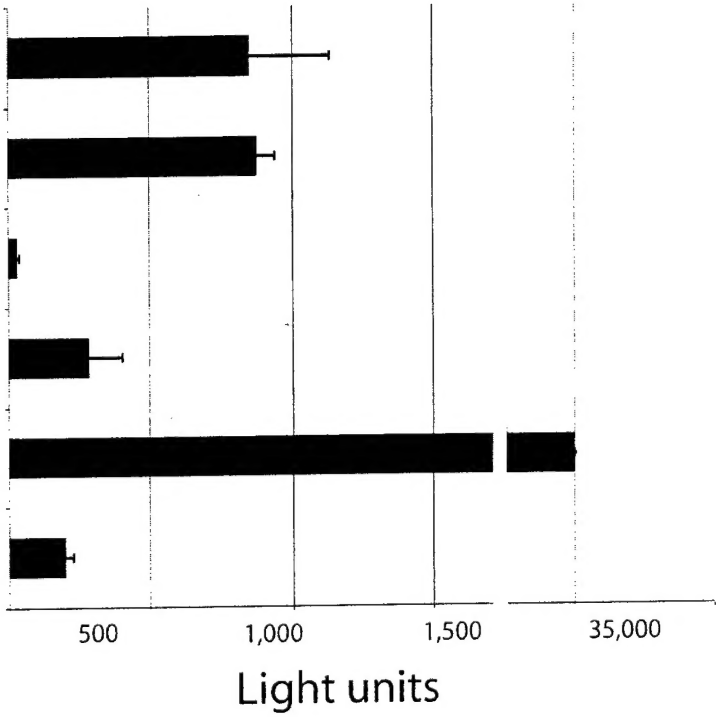
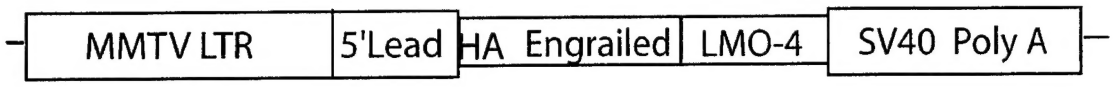
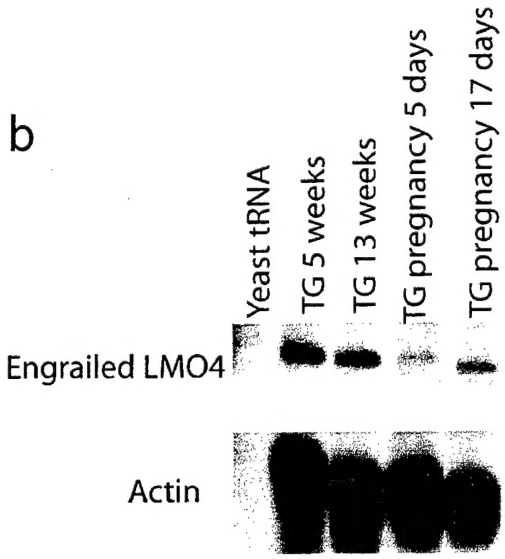


Fig. 3

a


MMTV LTR
5'Lead
HA Engrailed
LMO-4
SV40 Poly A

b


Yeast tRNA
TG 5 weeks
TG 13 weeks
TG pregnancy 5 days
TG pregnancy 17 days
Engrailed LMO4
Actin

c

d

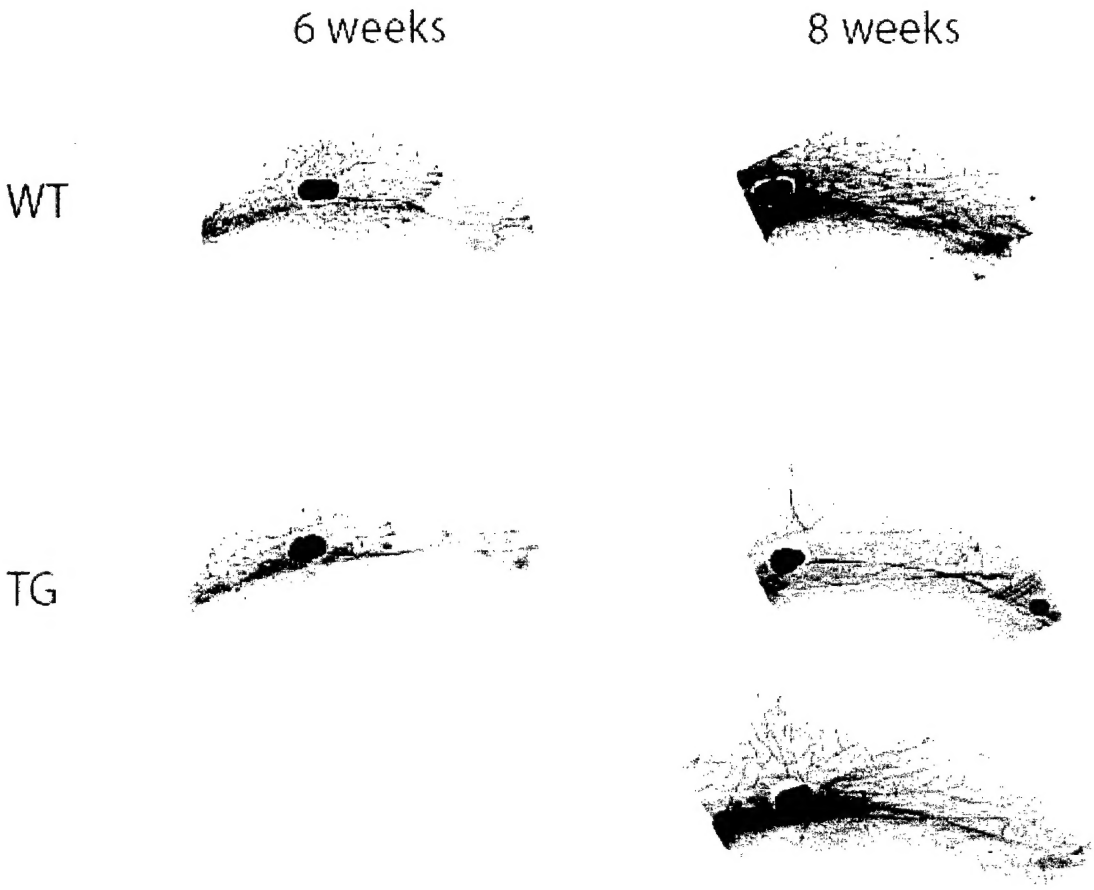
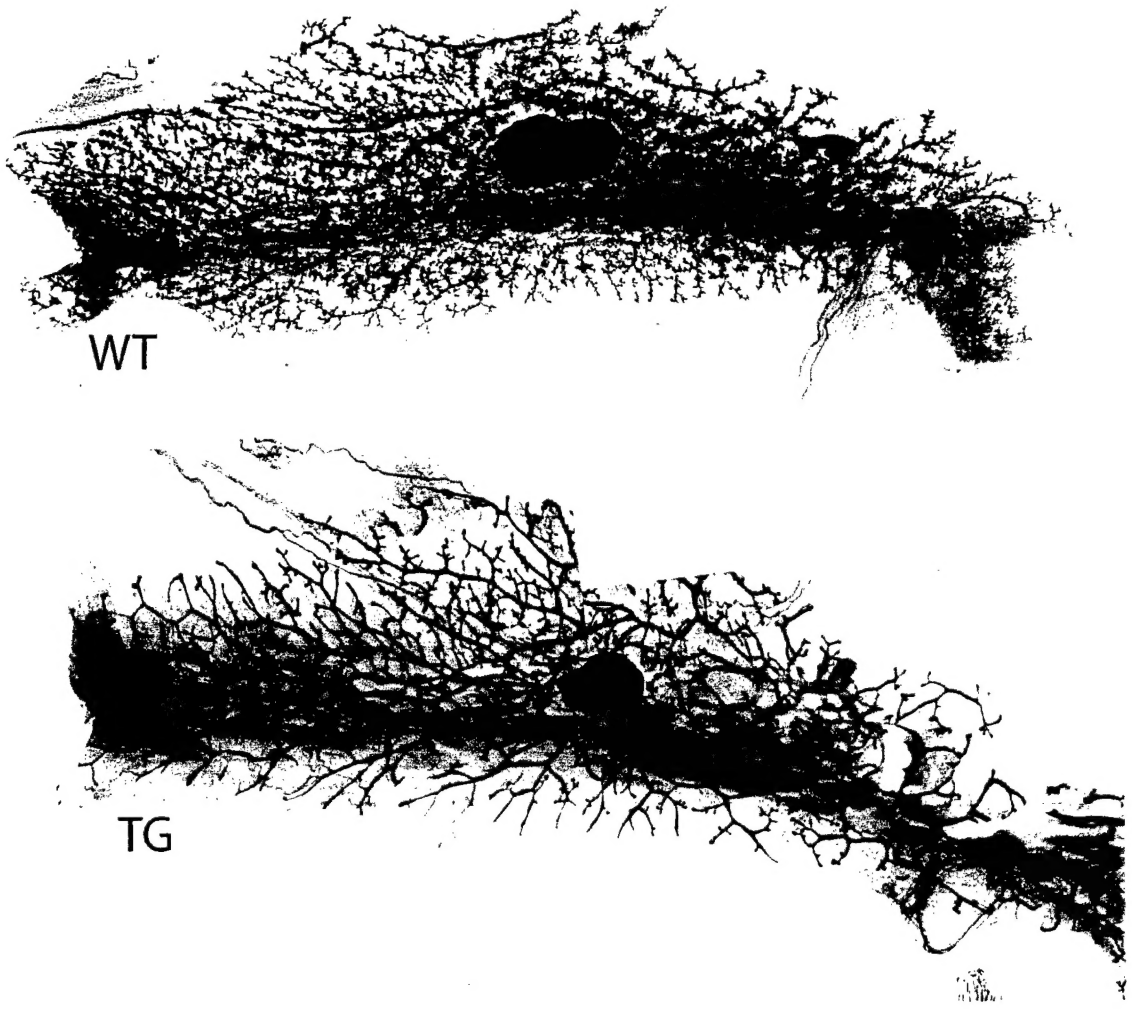

6 weeks
8 weeks
WT
TG

Fig. 4

a



b